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(Article begins on next page)

## Ischaemic preconditioning modulates the activity of Kupffer cells during *in vivo* reperfusion injury of rat liver

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This work was performed to elucidate further the main cellular events underlying the protective effect of ischaemic preconditioning in an *in vivo* rat liver model of 90 min ischaemia followed by 30 min reperfusion. A significant attenuation of the various aspects of post-ischaemic injury, namely necrosis and the levels of hydrogen peroxide and 5- and 15-hydroperoxyeicosatetraenoic acids, was afforded by the prior application of a short cycle of ischaemia/reperfusion (10 + 10 min) or when rats were previously treated with gadolinium chloride. However, when preconditioning was applied on Kupffer cell-depleted livers, no additional level of ischaemic tolerance was obtained. In terms of cellular pathology, this result could be suggestive of Kupffer cells as the target of the preconditioning phenomenon during the warm ischaemia/reperfusion injury. Accordingly, modulation of Kupffer cell activity was associated with a well-preserved hepatocyte integrity, together with low levels of pro-oxidant generation during reperfusion. As activated Kupffer cells can generate and release potentially toxic substances, their modulation by ischaemic preconditioning could help to provide new surgical and/or pharmacological strategies to protect the liver against reperfusion damage. Copyright © 2003 John Wiley & Sons, Ltd.

KEY WORDS — liver ischaemic preconditioning; Kupffer cell; oxidative stress

ABBREVIATIONS — HPETE, 5- and 15-hydroperoxyeicosatetraenoic acids; IP, ischaemic preconditioning; ROS, reactive oxygen species

### INTRODUCTION

Substantial evidence indicates that Kupffer cells play a central role in the pathogenesis of liver parenchymal cell damage during the early post-reperfusion phase, mainly through releasing ROS and other toxic mediators (i.e. cytokines) able to modulate hepatocyte metabolism and function.<sup>1,3–5</sup> In light of this and in order to improve liver function after reperfusion, various attempts have been made to block Kupffer cell activity, either through administration of specific toxins (e.g. GdCl<sub>3</sub>, dichloromethylene diphosphonate), methyl-

xanthine phosphodiesterase inhibitors (i.e. pentoxifylline) or by the use of calcium blocking agents (e.g. nisoldipine). However, the side-effects of these treatments cannot be entirely excluded.<sup>1,3–9</sup>

The term IP was coined to describe a protective mechanism against ischaemia–reperfusion injury and consists of previous short periods of ischaemia followed by reperfusion.<sup>10</sup> It was originally described in the heart, where it decreases infarct size after subsequent prolonged ischaemia and reperfusion.<sup>11</sup>

In the liver, the exact mechanisms involved in the status of cytoprotection conferred by IP against ischaemia/reperfusion injury are not fully understood. However experimental data suggest that modifications of the endothelin-1/nitric oxide ratio in favour of the latter, changes in energy metabolism, attenuation of *in situ* TNF- $\alpha$  production and release, as well as a blocking effect on the ROS-generating systems, are actively implicated.<sup>12–17</sup> Moreover, since the liver is composed

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of a heterogeneous cell population, it is likely that during preconditioning complex cross-talk could take place between parenchymal and non-parenchymal cells.

Accordingly, we investigated the effects of IP on Kupffer cell activity in a rat liver model of ischaemia/reperfusion injury.

## MATERIALS AND METHODS

### *Animal model*

Male Sprague-Dawley rats ( $224 \pm 5$  g body weight) were used; they had free access to standard chow diet and water. After anesthesia with 10% ketamine chlorhydrate solution, they were positioned under a heat lamp to maintain constant body temperature. After a midline laparotomy, selective inflow occlusion of the portal triad (hepatic artery, portal vein, and bile duct) to the left and median liver lobes was performed. Reperfusion was initiated by clamp removal. Samples from the post-ischaemic lobes were taken at the end of the reperfusion phase, and biochemical and morphological studies were carried out. All animals received humane care according to the criteria outlined in *Guide for the Care and Use of Laboratory Animals*.<sup>18</sup>

### *Depletion of Kupffer cells*

To inhibit Kupffer cell activity, a randomized set of rats were treated with gadolinium chloride ( $\text{GdCl}_3$ ;  $10 \text{ mg kg}^{-1}$  in sterile 0.9% NaCl solution, i.v.) 18 h before the ischaemia and reperfusion cycle.<sup>3</sup>

### *Experimental design*

The rats were subdivided into six groups, each group being of five animals.

**Group 1.** Sham saline: animals treated with sterile 0.9% NaCl solution were subjected to anaesthesia and laparotomy alone.

**Group 2.** Sham  $\text{GdCl}_3$ : as in group 1, but rats were treated with  $\text{GdCl}_3$  instead of sterile 0.9% NaCl solution.

**Group 3.** Standard Ischaemia/Reperfusion (Isch/Rep): rats were subjected to 90 min of ischaemia followed by 30 min of reperfusion.

**Group 4.** IP + Isch/Rep: before ischaemia/reperfusion, rats were subjected to 10 min ischaemia followed by 10 min reperfusion.<sup>17</sup>

**Group 5.**  $\text{GdCl}_3$  + Isch/Rep: before ischaemia/reperfusion, rats were treated with  $\text{GdCl}_3$ .

**Group 6.**  $\text{GdCl}_3$  + IP + Isch/Rep: before IP procedure, as in group 4, rats were treated with  $\text{GdCl}_3$ .

### *Biochemical assessment of liver cytotoxicity*

Irreversible hepatic damage was evaluated by determination of alanine aminotransferase and aspartate aminotransferase in blood-plasma obtained from the posterior cava vein after 30 min of reperfusion, using a commercial kit from Sigma Chemical Co. (St. Louis MO, USA).

### *$\text{H}_2\text{O}_2$ analysis*

Specimens from sham-operated and reperfused livers were homogenized (10%, w/v) in a buffer containing  $0.033 \text{ mol l}^{-1} \text{ Na}_2\text{HPO}_4$  and 0.9% KCl, pH 7.4 at  $4^\circ\text{C}$ . The homogenates were centrifuged at  $40,000 g$  for 45 min and the supernatant was used for  $\text{H}_2\text{O}_2$  analysis. The  $\text{H}_2\text{O}_2$ -mediated oxidation of  $\text{Fe}^{2+}$  to  $\text{Fe}^{3+}$  ion, under acidic conditions and in the presence of xylenol orange dye, was quantified as described elsewhere.<sup>19</sup> For the working reagent, 1 ml solution A containing ammonium ferrous sulphate (25 mM) and sulphuric acid (2.5 mM) was mixed with 100 ml of solution B containing sorbitol (100 mM) and xylenol orange ( $125 \mu\text{mol l}^{-1}$  in water. A 0.1-ml volume of supernatant was added to 1 ml of working reagent, mixed and incubated at room temperature for 15 min. The absorbance was read at 560 nm. The values were read against a standard curve prepared with 1 to  $50 \mu\text{mol l}^{-1}$  concentrations of  $\text{H}_2\text{O}_2$ . The absolute values of  $\text{H}_2\text{O}_2$  were expressed in  $\mu\text{mol l}^{-1}$ .

### *Determination of 5- and 15-HPETE*

The concentration of 5- and 15-HPETE in the lipid fraction from cytosol samples extracted by the method of Folch *et al.*<sup>20</sup> was determined by the method of Tordijman *et al.*,<sup>21</sup> slightly modified. Briefly, determination was by high performance liquid chromatography analyses with a Symmetry  $\text{C}_{18}$  column. The mobile phase used was a mixture of  $0.05 \text{ M NaH}_2\text{PO}_4$ , pH 5/methanol/acetonitrile (30:35:35, v:v). The HPETE levels in the samples were calculated by comparison with standard solutions of 5- and 15-HPETE (Sigma, St Louis, MO, USA). The results were expressed as  $\mu\text{M}$ .

### *Number of neutrophils*

Fragments from the left lateral lobe of about  $1.0 \times 0.5 \times 0.3 \text{ cm}$  were placed overnight in a solution of 4% formaldehyde in 50 mM phosphate buffer solution,

pH 7.4. Polymorphonuclear leukocytes (PMN) were identified on 5- $\mu$ m paraffin sections by means of the naphthol AS-D chloroacetate technique for esterase, following the method described by Moloney *et al.*<sup>22</sup> with slight modifications; the substrate was dissolved in dimethyl sulphoxide/Triton X-100 (9:1, v/v) and then 0.1% Fast Garnet dGBC in 0.1 M phosphate buffer solution, pH 8.5 was added. Red stained PMN were counted in 20 non-consecutive, randomly chosen  $\times 500$  histological fields. Results were expressed as the number of neutrophils per  $40 \times$  histological power field (hpf).

#### Protein determination

The protein content of the liver homogenates was determined by the spectrophotometric method described by Peterson<sup>23</sup> using bovine serum albumin as standard.

#### Statistical analysis

Data are presented as means  $\pm$  SE; the statistical significance of differences was analysed using the one-way analysis of variance (ANOVA) corrected by the Tukey–Kramer Multiple Comparisons test and with the Student *t* test where *p* values  $\leq 0.05$  were considered to be statistically significant.

#### RESULTS

In our experimental model, long-term ischaemia followed by reperfusion induced hepatic necrosis in both non-preconditioned and preconditioned animals. However, a marked reduction of irreversible cell injury was detectable when IP had previously been applied (Figure 1).

Among non-parenchymal liver cells, Kupffer cells are thought to constitute the major source of toxic mediators, namely ROS, released immediately after

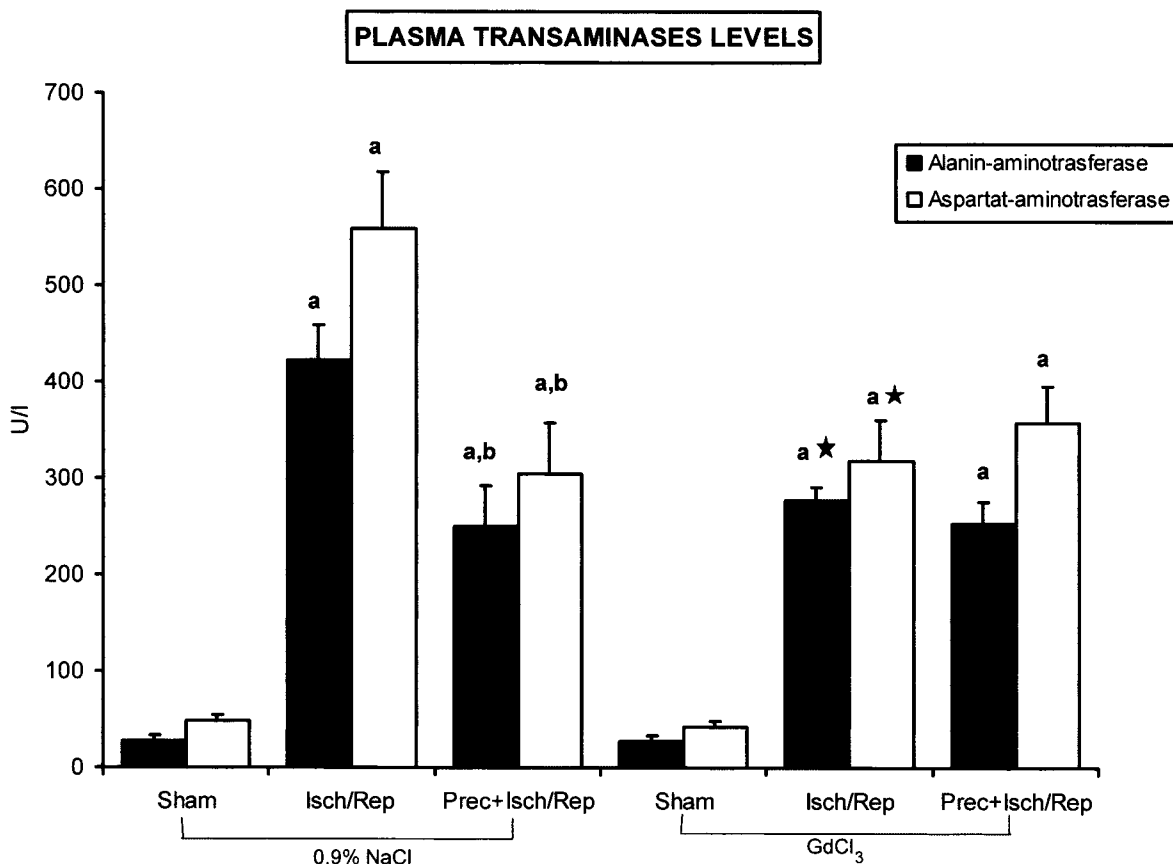


Figure 1. Ischaemic preconditioning and gadolinium chloride treatment attenuate the post-ischaemic irreversible damage of the liver. Data are presented as means  $\pm$  SE. *p* < 0.05; a versus sham; b versus Isch/Rep; ★ versus Isch/Rep from 0.9% NaCl-treated rats

reperfusion.<sup>1,3,6</sup> Cytolysis was also significantly attenuated in animals previously treated with GdCl<sub>3</sub> (Figure 1). However, the degree of ischaemic tolerance was not improved if preconditioning was previously applied to Kupffer cell-depleted rats (Figure 1).

In this study, we examined the changes in concentrations of H<sub>2</sub>O<sub>2</sub> and 5- and 15-HPETE during the course of the ischaemia/reperfusion injury, as by-products of the activity of the Kupffer cell's NADPH oxidase and 5-lipoxygenase.<sup>4,25,26</sup> To exclude neutrophils as possible sources of 5-lipoxygenase-derived hydroxyperoxides,<sup>27</sup> we evaluated the number of naphthol-AS-D-chloroacetate-positive infiltrating leukocytes.

After ischaemia and reperfusion, a 2.6-fold increase in the concentrations of H<sub>2</sub>O<sub>2</sub> was seen in the group of rats treated with 0.9% NaCl. This level of oxidative stress was reduced by 31% however, when the animals were previously submitted to GdCl<sub>3</sub> treatment (Table 1). Similarly, IP was able to protect against post-reperfusion generation of H<sub>2</sub>O<sub>2</sub> in the 0.9%

Table 1. Hepatic concentration of hydrogen peroxide after reperfusion (μmoles)

	Groups	
	0.9% NaCl treated	GdCl <sub>3</sub> treated
Sham	1.20 ± 0.08	1.40 ± 0.06
Isch/Rep	3.20 ± 0.09*	2.20 ± 0.17*†
Prec+Isch/Rep	1.70 ± 0.17*†	2.00 ± 0.17†

Data are presented as means ± SE.

*p* < 0.05; \*versus sham; †versus Isch/Rep; ‡versus Isch/Rep from 0.9% NaCl-treated rats.

NaCl-treated group (47% decrease). However, when the IP procedure was applied to the Kupffer cell-depleted livers, no additional attenuation over the level of H<sub>2</sub>O<sub>2</sub> production was observed (Table 1).

Similarly, GdCl<sub>3</sub> treatment and IP were able to attenuate the increased production of 5-lipoxygenase-derived lipid hydroperoxides following ischaemia/reperfusion (Figure 2). However, in the liver

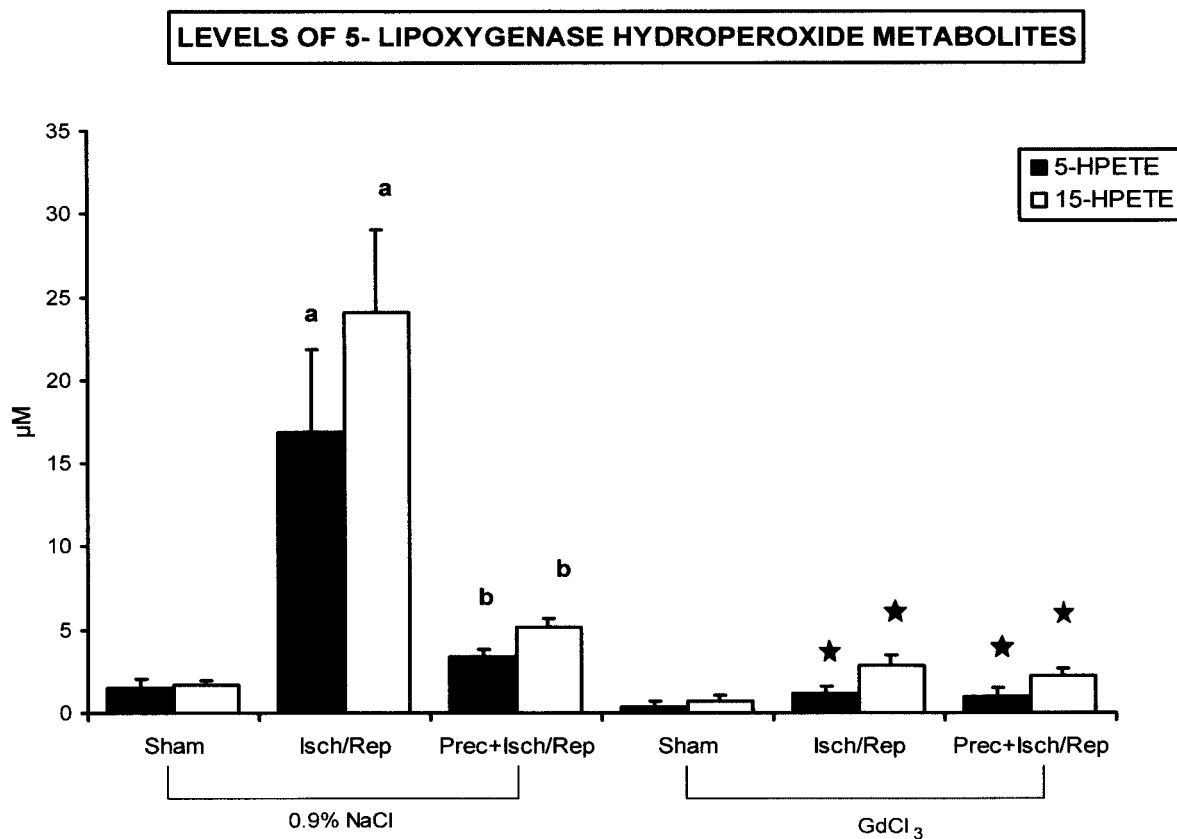


Figure 2. Ischaemic preconditioning and gadolinium chloride treatment attenuate the post-ischaemic generation of 5- and 15-hydroperoxyeicosatetraenoic acids. Data are presented as means ± SE. *p* < 0.05; a versus sham; b versus Isch/Rep; ★ versus Isch/Rep from 0.9% NaCl-treated rats

Table 2. Number of neutrophils in the liver after reperfusion (PMN/hpf)

	Groups	
	0.9% NaCl treated	GdCl <sub>3</sub> treated
Sham	2.0 ± 0.2	2.0 ± 1.0
Isch/Rep	7.5 ± 2.0*	7.5 ± 2.0*
Prec+Isch/Rep	10.0 ± 3.0*	8.5 ± 3.0*

Data are presented as means ± SE.

$p < 0.05$ ; \*versus sham.

depleted of Kupffer cells, IP did not accentuate the inhibitory effect over 5- and 15-HPETE production, so differences brought about by preconditioning of the liver cannot be discerned. Moreover, the fact that there was no change in the number of neutrophils seen in the reperfused livers allowed these cells to be ruled out as possible sources of 5- and 15-HPETE (Table 2).

## DISCUSSION

A prominent feature of ischaemia and reperfusion leading to liver damage is the activation of Kupffer cells.<sup>1,3,6</sup> Activated Kupffer cells are involved in the development of liver cell injury through production of ROS and inflammatory mediators, which directly contribute to the sinusoidal dysfunction and hepatocyte damage and death during the early phase of reperfusion, and indirectly by promoting neutrophil recruitment and initiation of inflammatory responses.<sup>28,29</sup> Modulation of Kupffer cell activity attenuated the early post-reperfusion damage. Accordingly, depletion of Kupffer cells with GdCl<sub>3</sub> was associated with a well-preserved hepatocyte integrity, together with low levels of pro-oxidant generation during reperfusion.

Products of lipid peroxidation, other than 4 hydroxy-2, 3-*trans*-nonenal and malondialdehyde, namely 5-lipoxygenase-derived lipid hydroperoxides, are also known to be toxic to cells.<sup>30</sup> As the enzyme 5-lipoxygenase is present only in the non-parenchymal cells of the liver, namely Kupffer cells,<sup>31</sup> measurement of its biological by-products at the end of the early phase of reperfusion studied here, could provide a functional index of Kupffer cell activity.

Are Kupffer cells targets of IP? A hallmark of this study is that application of IP to the livers allows us to reproduce practically identical findings to those obtained through GdCl<sub>3</sub> treatment, i.e. cytolysis and tissue oxidative stress were attenuated. However, when Kupffer cell-depleted livers were preconditioned, no additional level of tolerance against post-ischaemic damage was obtained. Moreover, the levels

of H<sub>2</sub>O<sub>2</sub> and 5- and 15-HPETE were almost identical to those obtained by either GdCl<sub>3</sub> treatment or IP alone. In our opinion, this fact constitutes evidence that a main mechanism of action of preconditioning against *in vivo* reperfusion injury could be the modulation of Kupffer cells activity. Accordingly, recent experiments from an isolated perfused rat liver model have shown that IP protects sinusoidal endothelial cells and suppresses Kupffer cell activation after cold storage and reperfusion.<sup>32,33</sup>

Could there be any mechanism(s) of cell damage not susceptible to modulation by IP? This question emerges because of the residual cytolysis and tissue oxidative stress found at the end of the reperfusion phase in both preconditioned and GdCl<sub>3</sub>-treated livers. A probable explanation for these results could be that neither IP nor GdCl<sub>3</sub> treatment are sufficient to attenuate the irreversible cell damage and the pro-oxidant levels generated during the sustained ischaemic phase.

The present study does not address the mechanisms by which IP offers tolerance against sustained ischaemia/reperfusion injury, but in terms of cellular pathology, it indicates Kupffer cells as a target of IP during this *in vivo* model of warm ischaemia/reperfusion injury to the liver. We would like to think that some mediator(s) formed during the brief cycle of ischaemia/reperfusion (10 min + 10 min) are able to modulate Kupffer cell activity. In agreement with this, adenosine has been implicated as an important mediator of cardiac and liver IP,<sup>13,34</sup> and it has been reported that adenosine is able to prevent activation of Kupffer cells and to suppress TNF- $\alpha$  release from them.<sup>15</sup>

During ischaemia, ATP is rapidly degraded to adenosine. Adenosine modulates several tissue functions through receptor-mediated mechanisms. Adenosine receptors differ in their biological and pharmacological responses to adenosine agonists and antagonists.<sup>35</sup> Adenosine A<sub>1</sub> receptors stimulate inhibitory G-proteins that block adenylate cyclase and decrease cAMP. By contrast, activation of A<sub>2</sub> receptors stimulates adenylate cyclase and increases cAMP. Accordingly, in recent experiments carried out with a cold storage model of liver injury, it was reported that adenosine mediates IP to sinusoidal endothelial cells through A<sub>2</sub> receptors and that adenosine A<sub>2</sub> receptor agonists, as well as cAMP, protect preconditioned sinusoidal endothelial cells against cold storage/reperfusion injury to the same extent as brief cycles of ischaemia-reperfusion (preconditioning phenomenon).<sup>36</sup>

Because adenosine suppresses endotoxin-stimulated tumour necrosis factor- $\alpha$  formation by Kupffer cells via an adenosine A<sub>2</sub> receptor pathway,<sup>37</sup> it seems

reasonable to believe that adenosine released by preconditioning interferes in some step(s) of the mechanism involved in the activation of the Kupffer cells' NADPH oxidase and 5-lipoxygenase enzymes, i.e. blocking the post-reperfusion release of toxic mediators:  $H_2O_2$  and 5- and 15-HPETE. Accordingly, it has been documented that intracellular cAMP-elevating agents have been shown to be associated with the downregulation of the oxidative burst and the inhibition of the 5-lipoxygenase translocation and activation in both neutrophils and cells of the macrophage/monocyte lineage.<sup>38–41</sup>

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